Failure of bacteriophage typing to detect an inter-hospital outbreak of methicillin-resistant *Staphylococcus aureus* (MRSA) in Zagreb subsequently identified by random amplification of polymorphic DNA (RAPD) and pulsed-field gel electrophoresis (PFGE)

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Objective: To establish the extent of inter-hospital spread of methicillin-resistant *Staphylococcus aureus* (MRSA) in Zagreb and to determine the most suitable method for typing local strains.

Methods: We analyzed a collection of 33 MRSA isolates from three Zagreb hospitals together with five unrelated British MRSA isolates by antibiogram typing, bacteriophage typing, randomly amplified polymorphic DNA (RAPD) analysis and pulsed-field gel electrophoresis (PFGE) after digestion with *Sma*I restriction endonuclease. Bacteriophage typing was done with the international set of *S. aureus* typing phages. RAPD and PFGE profiles were analyzed visually and by using the 'GelCompar' computer program.

Results: Antibiogram typing provided eight profiles. Thirty (91%) of the 33 Croatian strains of MRSA were non-typable by phage typing. Visual analysis of RAPD products identified six, and visual analysis of PFGE fragments nine, distinct profiles. Computer analysis of RAPD data separated British isolates from the Croatian ones, but did not cluster the visually determined RAPD types. PFGE computer analysis separated British isolates and clustered isolates in concordance with visual interpretation. Thirty-one of the 38 isolates (82%) were visually grouped in the same clusters by both molecular methods. The dominant strain was present in each of the three hospitals.

Conclusions: Bacteriophage typing was unhelpful for the analysis of Croatian MRSA, since most strains were untypable with the international set of bacteriophages. RAPD and PFGE were more successful in typing the organisms and showed evidence of inter-hospital spread of one predominant MRSA strain in all three Zagreb hospitals. Thus RAPD and PFGE proved to be a useful aid in elucidating the epidemiology of MRSA infection in Zagreb hospitals and should be established in Croatia for typing MRSA.

Key words: MRSA, inter-hospital transmission, phage typing, RAPD, PFGE

INTRODUCTION

Although methicillin-resistant *Staphylococcus aureus* (MRSA) outbreaks were described in some Croatian hospitals in the late 1980s [1], the prevalence of MRSA in Croatia remained low until 1990. In recent years, however, MRSA has become an epidemic problem in many large hospitals throughout the country and in Zagreb we suspect that strains of MRSA are spreading between hospitals. At present, methods for typing staphylococci are not routinely available in Croatia, apart from antibiogram typing. However, this method may be unstable and is often not discriminatory enough to determine epidemiologic relationships among MRSA isolates.

In order to establish the extent of inter-hospital spread of MRSA and to determine which typing method would be most suitable for typing local strains, we typed a collection of 33 MRSA isolates from three different Zagreb hospitals by four methods. These were antibiotyping, phage typing, and two genetic typing methods—randomly amplified polymorphic DNA (RAPD) analysis and pulsed-field gel electrophoresis

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(PFGE). The RAPD analysis uses short primers of an arbitrary sequence to amplify genomic DNA in a low-stringency polymerase chain reaction (PCR) [2]. These primers hybridize with complementary chromosomal sequences that vary among different strains, and therefore produce different amplification products which can be separated by gel electrophoresis to produce characteristic 'fingerprints'. PFGE involves the separation of whole cell DNA digests with low-frequency cleavage site restriction endonucleases and provides 'fingerprints' with fewer well-separated bands [3].

MATERIALS AND METHODS

MRSA isolates

In total, 33 isolates of MRSA were collected from clinical specimens from 33 patients from three Zagreb hospitals in February 1995. Isolates were obtained from deep and superficial surgical wounds (24 isolates), tracheal aspirates (three), gastric aspirate (one) and an intravenous catheter tip (one). Twenty-three isolates were collected from the Clinical Hospital Center Rebro, a 1200-bed teaching hospital; seven from the Sveti Duh Hospital, a 700-bed general hospital; and three from the University Hospital of Traumatology, a 220-bed trauma hospital. The majority of the MRSA isolates (28) were from surgical and orthopedic patients. All MRSA isolates were initially isolated on 5% blood agar plates and identified as *Staphylococcus aureus* by morphology and the tube coagulase test. All the strains were tested for sensitivity to antibiotics and typed by phage typing, RAPD and PFGE. Five previously identified phage non-typable isolates from St Thomas' Hospital, London, UK were included in the molecular analyses to act as unrelated control strains.

Antibiotic susceptibility testing

Antibiotic susceptibility testing was performed by a disk diffusion method [4] for gentamicin, erythromycin, clindamycin, rifampicin, tetracycline, co-trimoxazole, fucidic acid, ciprofloxacin, netilmicin, mupirocin, neomycin and vancomycin. Susceptibility to methicillin was determined by using a 5-μg methicillin disk on a blood agar plate incubated at 30°C for 24 h; an inhibition zone of ≤5 mm width from the edge of the disk was considered to indicate resistance. Isolates were grouped according to the antibiogram profiles.

Phage typing

Bacteriophage susceptibility testing was kindly performed by the Central Public Health Laboratory, Colindale, London with the international set of *Staphylococcus aureus* typing phages.

RAPD typing

DNA isolation

DNA was isolated as described by Ausubel et al [5], except that pelleted bacterial cells were first treated with 50 μL of lysostaphin (50 mg/L) and 100 μL of lysozyme (2 mg/mL) (Sigma Chemical Co, Poole, UK) for 1 h at 37°C.

PCR amplification

We used a combination of two 10-mer primers EP 015 (5′-ACA ACT GCT C-3′) (prepared by the Advanced Biotechnology Centre, The Charing Cross and Westminster Medical School, London) and KAY1 (5′-AGC AGC CTG C-3′) (supplied by Pharmacia Biotech, Uppsala, Sweden). For each sample we used 200 μM of each nucleotide (Sigma, Amersham, UK), 5 μL of 10× reaction buffer, 500 nM primer, 50 ng of template DNA, 2 U of *Taq* XL DNA polymerase (Northumbria Biochemicals LTD, CramLington, UK), made up to 50 μL with molecular biology-grade water. This mixture was overlaid with 50 μL of mineral oil. The PCR cycling profile is described in Table 1. Reactions were carried out in a Hybaid Thermal Reactor (Hybaid, Teddington, UK). The products of the RAPD analysis were electrophoresed on 2% agarose gels in TBE buffer. Synthetic PCR molecular weight markers (Cambridge Bioscience LTD, Cambridge, UK) were run on each gel. The gels were run for 5–6 h at 60 V to allow complete separation of bands. DNA profiles were visualized by UV light after ethidium bromide staining, and photographed with a red filter and Polaroid 667 film.

PFGE typing

Plugs containing *Staphylococcus aureus* DNA were prepared as described previously [6] and digested with *Sma*I restriction endonuclease (MBI Fermentas, Vilnius, Lithuania). These *Sma*I digests were loaded onto a 1% agarose gel and subjected to electrophoresis in a DrII Chef electrophoresis system (Bio-Rad, Hemel Hempstead, UK) for 24 h, with a pulse time ramped from 10 to 100 s, at 180 V and 14°C throughout.

Analysis of the gels

Gels were visually analyzed blind by three independent individuals. Discrepancies were resolved by consensus. Profiles were considered different if they differed by two or more bands for RAPD and by three or more bands for PFGE [7]. Profiles varying by a single band for RAPD and up to two bands for PFGE were identified as subtypes. Types were designated by capital letters, and subtypes by primes.

The gels were also subjected to computer analysis by using the 'GelCompar' computer program (Applied

Table 1 List of MRSA isolates typed by phage, antibiogram, and visual analysis of RAPD and PFGE profiles

Strain	Source	Phage type	Antibiotype	RAPD	PFGE
1	SDH/surg	NT	A	A	A
2	SDH/surg	NT	B	B	B
3	SDH/surg	NT	B	B	B
4	SDH/surg	NT	C	B	B
5	SDH/ort	NT	C	B′	B′
6	SDH/int	NT	C	C	C
7	SDH/nephr	NT	A	D	D
8	TR	NT	C	B	B″
9	TR	NT	D	B	E
10	TR	III (47,85/42,54,75,77)	C	E	C
11	KBC/surg	NT	C	B	B
12	KBC/surg	NT	C	B	B
13	KBC/surg	NT	C	B	B
14	KBC/surg	NT	C	B	B
15	KBC/surg	NT	C	B	B
16	KBC/surg	NT	C	B	B
17	KBC/surg	NT	C	B	B
18	KBC/surg	NT	C	B	B
19	KBC/surg	NT	C	B	B
20	KBC/surg	NT	C	B	B
21	KBC/surg	NT	C	B	B
22	KBC/surg	NT	C	B	B
23	KBC/surg	NT	C	B	B
24	KBC/surg	NT	C	B	B
25	KBC/surg	NT	C	B	B
26	KBC/surg	III (47,54,75,85,81/83A)	C	E	C
27	KBC/int	NT	C	B	B
28	KBC/ped surg	NT	D	B	B
29	KBC/ped surg	III (77/77,84)	E	F	F
30	KBC/neuro	NT	C	B	B
31	KBC/neuro	NT	C	A′	G
32	KBC/card	NT	A	B″	A′
33	KBC/card	NT	A	B″	A′
34	STH UK	NT	G	E	H
35	STH UK	NT	G	E	H
36	STH UK	NT	C	E	I
37	STH UK	NT	H	E	H
38	STH UK	NT	G	E	H

SDH, Sveti Duh Hospital; TR, Trauma Hospital; KBC, Clinical Center Rebro; surg, surgery; int, intensive care; nephr, nephrology; ped surg, pediatric surgery; neuro, neurosurgery; card, cardiology; STH UK, St Thomas' Hospital, London, UK.
NT, bacteriophage non-typable.
Strains resistant to following antibiotics:
A—gentamicin, tetracycline, erythromycin, fucidic acid, neomycin, ciprofloxacin.
B—gentamicin, tetracycline, erythromycin, clindamycin, rifampicin, neomycin, ciprofloxacin, netilmicin.
C—gentamicin, tetracycline, erythromycin, clindamycin, rifampicin, neomycin, ciprofloxacin.
D—gentamicin, tetracycline, erythromycin, clindamycin, neomycin, ciprofloxacin.
E—gentamicin, tetracycline, rifampicin, neomycin, ciprofloxacin.
F—gentamicin.
G—gentamicin, erythromycin, clindamycin, rifampicin, neomycin, ciprofloxacin, mupirocin.
H—gentamicin, erythromycin, clindamycin, rifampicin, neomycin, ciprofloxacin.

Maths, Kortrijk, Belgium). Polaroid photographs of the gels were scanned and saved as 256 color 'TIF' files. These images were normalized, the background was removed using the 'rolling-disk method', a similarity matrix was produced by the Dice coefficient, and a dendrogram was constructed from the resulting data by the unweighted pair-group method averaged (UMPGA).

RESULTS

Bacteriophage typing

Thirty (91%) of the 33 Croatian strains of MRSA were non-typable by phage typing. The three typable strains belonged to phage group III. Two of these three strains shared the same antibiotic, RAPD and PFGE profile and expressed the following phage patterns: 47,85/

42,54,75,77 and 47,54,75,85,81/83A. The third strain had the phage pattern 77/84 and had unique antibiogram, RAPD and PFGE profiles.

Antibiogram typing

Among the 33 Croatian MRSA isolates, four had the antibiogram profile A, two profile B, and 24 profile C. There was one isolate each with profile D, E and F. The five British MRSA isolates had two additional antibiogram profiles, G and H (Table 1).

RAPD typing

Visual analysis of RAPD products identified six (A–F) distinct profiles (Table 1 and Figure 1). However, although computer analysis of the RAPD profiles separated the British MRSA isolates into a separate group, the Croatian isolates were distributed into a series of clusters that were unrelated to their origin or to the visual RAPD grouping (Figure 2).

PFGE typing

Visual analysis of PFGE fragments identified nine (A–I) distinct profiles (Table 1 and Figure 3). Computer analysis of PFGE profiles grouped the British isolates in a single cluster which was separated from the other strains. All the visual PFGE type B isolates formed a single cluster, and the visual types A, C, D, E, F and G were all clustered into separate groups (Figure 4).

Table 2 MRSA profiles as determined by visual analysis for RAPD and PFGE

RAPD profile	PFGE profile								
	A	B	C	D	E	F	G	H	I
A	1						1		
B	2	23			1				
C			1						
D				1					
E			2					4	1
F						1			

Correlation between typing methods

Antibiotic profiles correlated with visual analysis of RAPD profiles in 66.7% of strains and with PFGE profiles in 75.8% of strains (Table 1). RAPD and PFGE together discriminated 11 electrophoretic profiles (Table 2). Thirty-one of the 38 isolates (82%) were visually grouped in the same clusters by both methods (Table 1). Strains 9, 10, 26, 31, 32, 33 and 36 were clustered differently by RAPD and PFGE (Table 1). The dominant B/B strain was present in each of the three hospitals.

DISCUSSION

MRSA is a frequent nosocomial pathogen, and typing systems for differentiating among strains of MRSA are

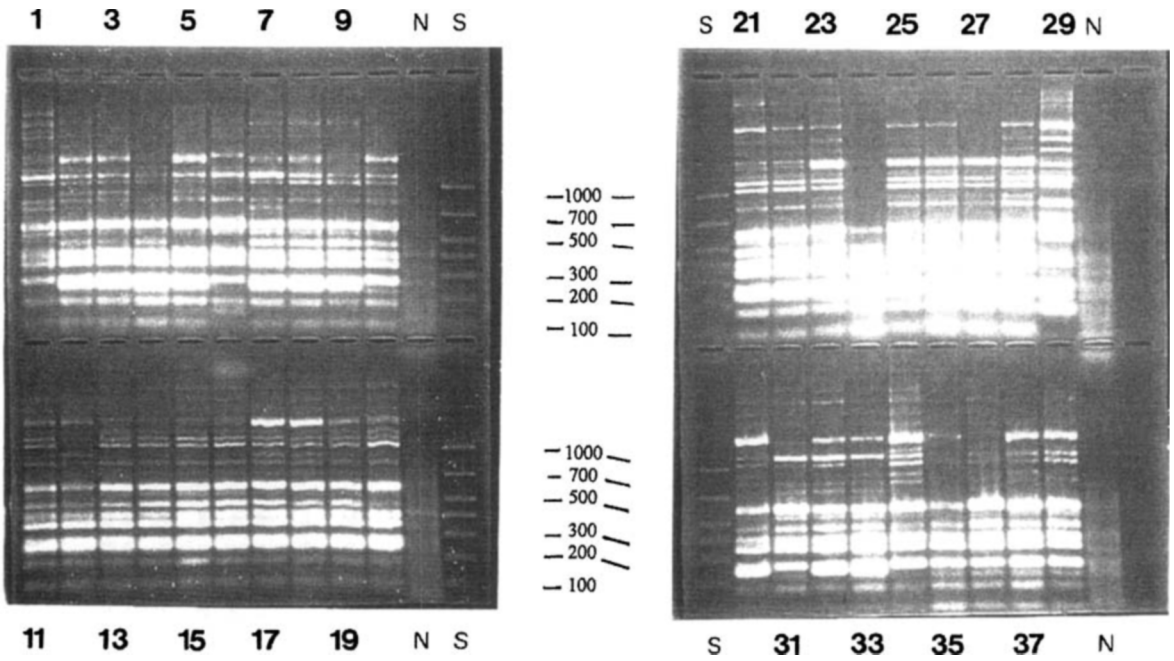

Figure 1 RAPD profiles for the 38 MRSA isolates. The isolates are arranged in gels in numerical order according to Table 1. Numbers of odd lanes are indicated. N, negative control; S, standard DNA (sizes are indicated in bp).

necessary to obtain information about sources and routes of transmission of these organisms. Sensitivity to antibiotics is a simple, readily available typing method that may signal the presence of a new strain and draw attention to an outbreak in routine clinical work [8–10]. However, susceptibility patterns may vary among isolates of the same clone, since many antibiotic resistance genes are plasmid-borne and may easily be

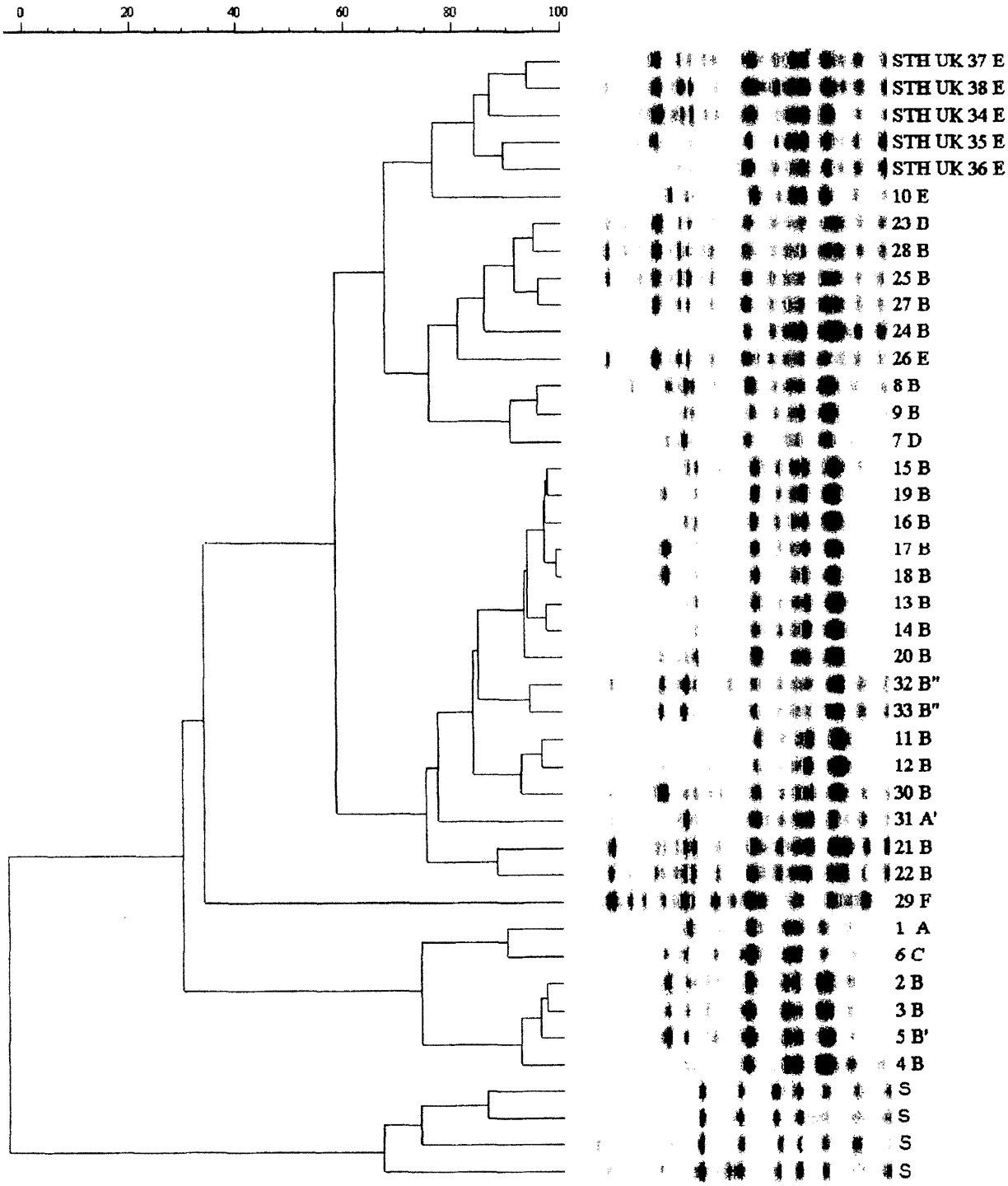

Figure 2 UMPGA dendrogram derived from RAPD profiles. Numbers indicate isolate identity; types determined by eye are indicated by capital letters. S, size marker (corresponds to Figure 1).

gained or lost during the course of an outbreak [11,12]. Furthermore, the sensitivity pattern may be poorly discriminative [9,11,13,14], since the use of antibiotics favors the emergence of resistance concurrently in independent strains. In our study, antibiotic profiles did not correlate well with RAPD or PFGE types. Where two or more isolates of MRSA had the same RAPD and PFGE profile and differed in sensitivity to only one antibiotic, it is likely that these strains were related but had acquired or lost resistance to the antibiotic.

Bacteriophage typing is still a mainstay in the epidemiologic analysis of *Staphylococcus aureus* infections [15–17]. It is a simple and well-studied method [15], but due to problems of reproducibility, it is best performed in reference laboratories, and the results are most reliable when all isolates to be compared are tested simultaneously [11]. A limitation of this method is that some strains may be non-typable [11,18]. In this study, phage typing was unhelpful for distinguishing among Croatian strains, as 91% of them were non-typable by phage.

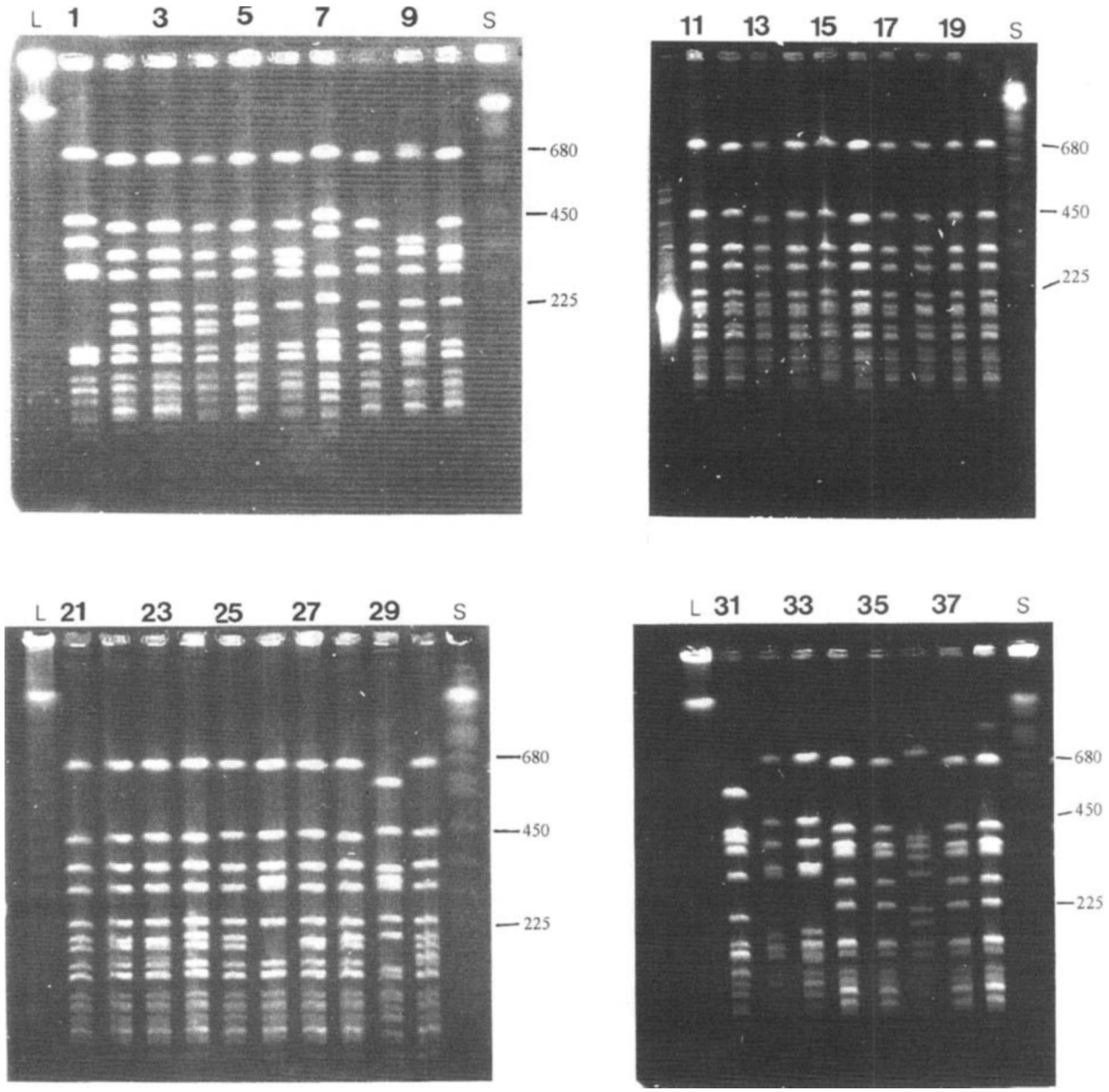

Figure 3 PFGE profiles for the 38 MRSA isolates. The isolates are arranged in gels in numerical order according to Table 1. Numbers of odd lanes are indicated. S, *Saccharomyces cerevisiae* DNA (sizes are indicated in kbp). L, Lambda concatomers.

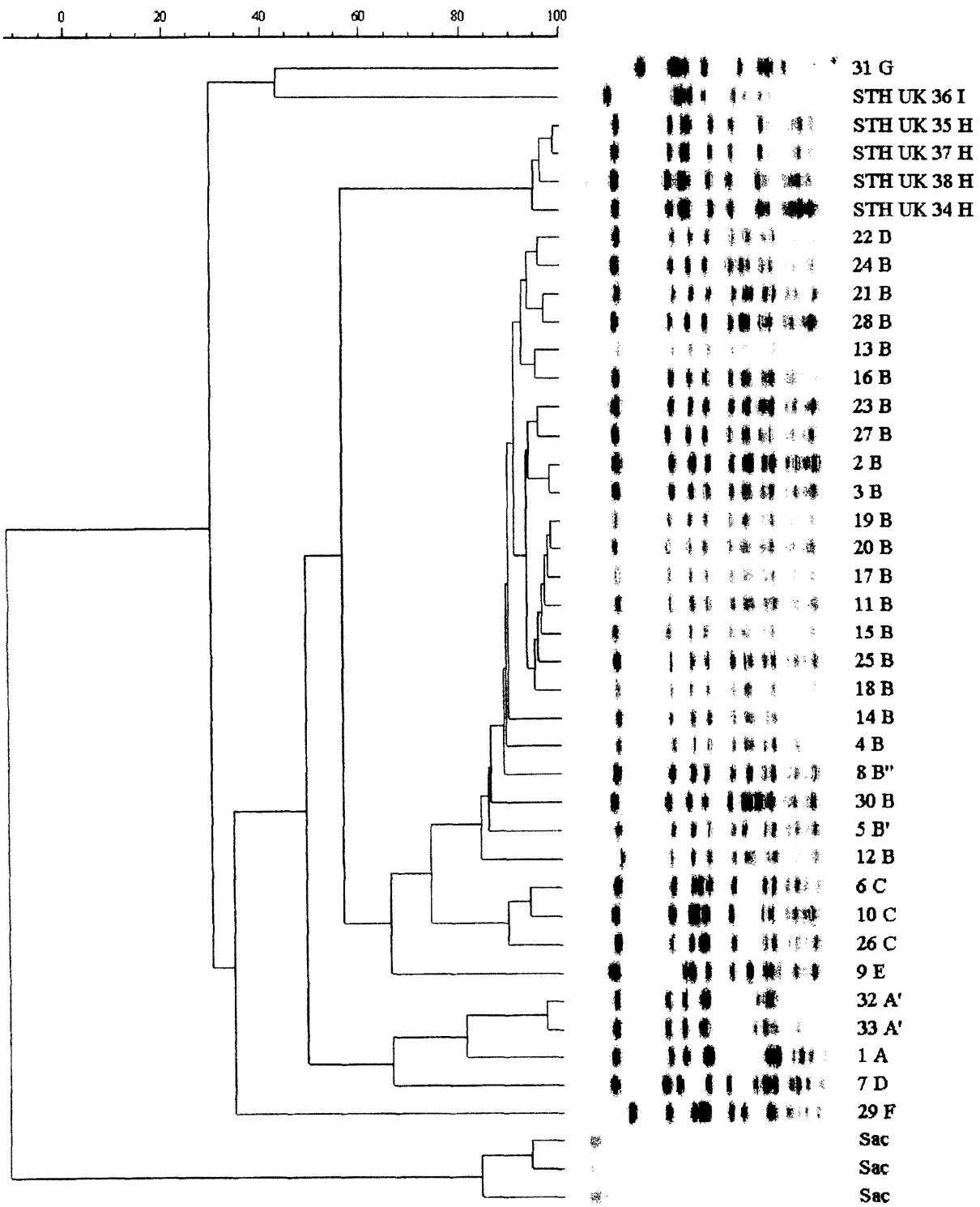

Figure 4 UMPGA dendrogram derived from PFGE profiles. Numbers indicate isolate identity; types determined by eye are indicated by capital letters. Sac, size marker (corresponds to Figure 3).

As genotyping techniques become more readily available and easier to perform, they are being more frequently applied to describe local outbreaks. A variety of chromosomal DNA analysis techniques have been used to type MRSA [11]. In this study, we used PFGE after restriction of genomic DNA with *SmaI*. This method is highly reproducible and has been shown to have good discriminatory power [3,13,18–20]. However, it takes a few days to obtain results by this method, and this is a limitation for epidemiologic investigation. Therefore, we also typed the strains by RAPD, a rapid and technically not too demanding method that does not require specialized electrophoresis equipment, which we have used in previous studies of British and Croatian MRSA [6,21]. This analysis, also called arbitrarily primed PCR (AP-PCR), has shown good discriminatory power in typing isolates of MRSA, especially when several primers or primer pairs are used in combination [16]. In this study we used a pair of primers that we had successfully used in a previous study [6].

While Hojo et al found that RAPD typing correlated well with PFGE results [22], Saulnier et al reported that RAPD was less discriminatory than PFGE for typing MRSA [23]. In our collection of 38 strains, we obtained six different profiles by visual analysis of RAPD gels and nine by PFGE. The combination of both methods, however, provided greater discrimination than either used alone. The unrelated phage non-typable isolates from the UK were distinguished by both molecular methods from the Zagreb phage non-typable isolates, but by RAPD analysis these isolates were indistinguishable from two of the group III strains. Both visual analysis of RAPD and PFGE profiles produced clusters that showed a high degree of correlation.

In this study, the PFGE profiles were easier to interpret than the RAPD profiles, because they consisted of well-separated bands of consistent intensity. Thus, although easier to perform, RAPD analysis produced profiles that were more difficult to interpret by eye. Furthermore, it was difficult to produce meaningful dendrograms from the RAPD profiles using the software available in our laboratory (GelCompar). Although the dendrogram produced from the RAPD profiles clearly separated the unrelated British phage non-typable isolates, the profile B outbreak strains were randomly distributed throughout the remainder of the dendrogram (Figure 2). Thus, computer analysis of the RAPD data was not helpful epidemiologically. In contrast, the clusters produced by computer analysis of the PFGE profiles correlated well with those identified by eye (Figure 4).

The two DNA-based techniques in combination provided strong evidence that a single type (designated B with both methods) was predominant in all the three Zagreb hospitals. In all three hospitals, the majority of MRSA strains were isolated from surgical and orthopedic patients, suggesting that the transfer of patients or staff between hospitals may have been responsible for inter-hospital spread. The results of this study suggest widespread transmission of MRSA among hospitals in Zagreb, stressing the need to improve inter-hospital communication and infection control measures for surgical patients transferring between hospitals [21].

Bacteriophage typing was unhelpful for the analysis of Croatian MRSA, since most strains were untypable with the international set of bacteriophages. Since molecular methods proved more successful, it does not seem worthwhile to investigate alternative phages for use in Croatia. In contrast, RAPD and PFGE proved useful for elucidating the epidemiology of MRSA infection in our hospitals and should be established for typing Croatian MRSA.

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